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A Complex Ergovaline Gene Cluster in Epichloë Endophytes of Grasses▽†

Damien J. Fleetwood,[1,2] Barry Scott,[2] Geoffrey A. Lane,[1] Aiko Tanaka,[2] and Richard D. Johnson[1]*

AgResearch, Grasslands Research Centre, Palmerston North, New Zealand,[1] and Centre for Functional Genomics, Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand[2]

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Clavicipitaceous fungal endophytes of the genera *Epichloë* and *Neotyphodium* form symbioses with grasses of the subfamily Pooideae, in which they can synthesize an array of bioprotective alkaloids. Some strains produce the ergopeptine alkaloid ergovaline, which is implicated in livestock toxicoses caused by ingestion of endophyte-infected grasses. Cloning and analysis of a nonribosomal peptide synthetase (NRPS) gene from *Neotyphodium lolii* revealed a putative gene cluster for ergovaline biosynthesis containing a single-module NRPS gene, *lpsB*, and other genes orthologous to genes in the ergopeptine gene cluster of *Claviceps purpurea* and the clavine cluster of *Aspergillus fumigatus*. Despite conservation of gene sequence, gene order is substantially different between the *N. lolii*, *C. purpurea*, and *A. fumigatus* ergot alkaloid gene clusters. Southern analysis indicated that the *N. lolii* cluster was linked with previously identified ergovaline biosynthetic genes *dmaW* and *lpsA*. The ergovaline genes are closely associated with transposon relics, including retrotransposons and autonomous and nonautonomous DNA transposons. All genes in the cluster were highly expressed in planta, but expression was very low or undetectable in mycelia from axenic culture. This work provides a genetic foundation for elucidating biochemical steps in the ergovaline pathway, the ecological role of individual ergot alkaloid compounds, and the regulation of their synthesis in planta.

Fungal endophytes of the genera *Epichloë* and anamorphic *Neotyphodium* (Clavicipitaceae, Ascomycota) are obligate biotrophs that form symbioses with grasses of the subfamily Pooideae (45). Phylogenetic analysis suggests that the asexual *Neotyphodium* species are generally hybrids of the sexual *Epichloë* species (35). Some species, however, are direct asexual derivatives of sexual species; *Neotyphodium lolii*, for example, is a derivative of *Epichloë festucae* (35). The fungi colonize the intercellular spaces of all the aboveground parts of the plant, including the reproductive tissues, but do not infect the roots (14). Endophyte infection confers several ecological benefits to infected plants, including resistance to invertebrate and vertebrate herbivory as well as enhanced growth, mineral uptake, and resistance to drought (17, 32). Resistance to herbivory is conferred to host plants by fungus-produced secondary metabolite alkaloids (10). Four classes of epichloë alkaloids have been characterized: indole diterpenes, lolines, peramine, and the ergot alkaloids (10).

Ingestion of ergot alkaloids by livestock grazing on endophyte-infected pastures can cause toxic effects, including poor weight gain, hyperthermia, convulsions, reduced fertility, gangrene of the extremities, and death (2, 50). Losses as a result of ergot alkaloid poisoning are significant and a major cost to the global agricultural industry (29). The effects of ergot alkaloid poisoning are attributed mainly to the ergopeptine end product, ergovaline (2). However, transport across ruminant gastric membranes is much higher for intermediate lysergyl compounds than for ergopeptines, suggesting that intermediate ergot alkaloids may have a significant role (28).

Ergot alkaloids are produced by ascomycetous fungi from discontinuous taxonomic groupings, including plant-associated fungal genera from the family Clavicipitaceae and some members of the order Eurotiales, including the human pathogen *Aspergillus fumigatus* (46). Much of the chemistry for ergot alkaloid synthesis has been elucidated from the ergot fungus, *Claviceps purpurea*. The first committed step is the formation of dimethylallyl tryptophan (DMAT) catalyzed by DMAT synthase using primary metabolites tryptophan and dimethylallyl diphosphate (23). The gene *dmaW*, encoding DMAT synthase, has been characterized from *Claviceps fusiformis* (52), *C. purpurea* (53), *A. fumigatus* (19), and the endophyte *Neotyphodium* sp. strain Lp1 (*Epichloë typhina* × *N. lolii*) (57). Serial redox reactions form clavine intermediate compounds chanoclavine, agroclavine, and elymoclavine (22), with the latter converted to D-lysergic acid by a cytochrome P450 encoded by the *cloA* gene, which was recently characterized for *C. purpurea* (26). D-Lysergic acid can then be converted into several lysergyl amides and the ergopeptines (22).

Ergopeptines are formed by the nonribosomal peptide synthetase (NRPS)-catalyzed addition of a tripeptide to activated lysergic acid (56). NRPSs are large multimodular enzymes where, generally, one module activates and tethers one specific amino acid or carboxylic acid substrate and catalyzes peptide bond formation with a substrate activated and bound to an upstream module, and so on, resulting in small peptides with defined sequences (34). In ergopeptine synthesis, as elucidated for *C. purpurea*, lysergic acid is activated and tethered to the LpsB (LPS2) enzyme, a single-module NRPS (18, 42). The lysergyl group is then added to a specific amino acid tethered

* Corresponding author. Mailing address: AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand. Phone: 64 6 351 8090. Fax: 64 6 351 8032. E-mail: richard.johnson@agresearch.co.nz.

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to the first module of LpsA (LPS1), a three-module NRPS (42). Two further specific amino acids are sequentially added, and the tripeptide is heterocyclized and released from LpsA as lysergyl peptide lactam (56). One further heterocyclization step occurs to yield the final ergopeptine product (40). The tripeptide moiety in ergovaline, the most abundant epichloë-produced ergopeptine, consists of alanine, valine, and proline (8). The genes encoding the LPS1 and LPS2 enzymes, *lpsA* and *lpsB*, have been cloned from *C. purpurea* (18, 25, 53), and the *lpsA* gene has been characterized from *Neotyphodium* sp. strain Lp1 (20, 38).

Genes for natural product biosynthesis are often found clustered in fungal genomes, and gene clusters for ergot alkaloid biosynthesis have been identified for *C. purpurea* (25, 53) and *A. fumigatus* (19, 54); they contain 13 and 14 genes, respectively. Putative functions have been proposed for the uncharacterized genes in the clusters based on bioinformatics; however, their conclusive roles in ergot alkaloid synthesis await functional analyses. Genes shared between the two clusters are presumed to be responsible for the early steps common to the two organisms, while genes unique to *C. purpurea* are proposed to be required for lysergic acid and ergopeptine biosynthesis and genes found only in the *A. fumigatus* cluster presumably are involved in the clavine decorations unique to that organism (19). Although the *dmaW* and *lpsA* genes have been cloned from epichloë species (20, 38, 57), additional biosynthetic genes assumed to be clustered were not isolated.

This study arose from the isolation, by degenerate PCR, of an *N. lolii* NRPS fragment, ps12, which is preferentially expressed during biotrophic growth (48). In this work, we show that the full-length gene associated with ps12 is the *lpsB* gene involved in ergovaline biosynthesis. Having isolated *lpsB*, our further objectives were (i) to determine whether ergot alkaloid biosynthetic genes were clustered in *N. lolii* by using chromosome walking and Southern blot analysis, (ii) to confirm the role of *lpsB* in ergot alkaloid biosynthesis by using targeted deletion and complementation, and (iii) to determine whether the genes in the cluster were preferentially expressed in planta, concurrent with ergot alkaloid production.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used in this study, XL1-Blue (9), KW251 (Promega Corp. Madison, WI), SOLR (Stratagene, La Jolla, CA), and Top 10 (Invitrogen Corp., Carlsbad, CA), were grown on Luria-Bertani agar plates supplemented, where necessary, with either ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

Fungal strains and growth conditions. Experiments were performed with *N. lolii* strain Lp19 (15) and *E. festucae* strain Fl1 (61). Fungal cultures were grown and maintained as described previously and supplemented where necessary with hygromycin (150 μg/ml) or Geneticin (220 μg/ml). Cultures of *E. festucae* Fl1 used for expression analysis were grown on either potato dextrose (PD) medium or one of two defined media, Mantle A (33), with high (2.0 g/liter) or low (0.2 g/liter) KH_2PO_4, or Czapek-Dox salts plus nitrogen (100 mM NH_4Cl) and/or a carbon source (100 mM glucose). Cultures incubated in plant extract were first grown in PD broth for three days and then filtered through sterile Whatman 3MM paper and added to the extract for 30 min. The plant extract was prepared essentially using the method of Lev et al. (31), and endophyte-free perennial ryegrass tillers were ground to a fine powder under liquid nitrogen and mixed 1:1 (wt/vol) with sterile distilled H_2O. The suspension was centrifuged at 13,000 rpm for 5 min, and the supernatant was removed for use.

Chemicals. All solvents used for extractions and high-performance liquid chromatography (HPLC) were HPLC grade. Ergotamine tartrate was obtained from Sigma-Aldrich Co. (St. Louis, MO). Authentic ergovaline was provided by F. Smith, Auburn University. Authentic samples of lysergic acid and ergine were provided by M. Flieger (Czech Academy of Sciences).

Construction and screening of libraries. A λZAPII library (Stratagene) was constructed by partially digesting *N. lolii* Lp19 genomic DNA with Tsp509I and purifying from an agarose gel in the 2- to 7-kb size range. This DNA was then ligated into an EcoRI-digested λZAPII insertion vector and packaged using Gigapack III packaging extract (Stratagene) per the manufacturer's instructions. Clones were also obtained from a previously described λGEM-12 library to *N. lolii* Lp19 (61). Libraries were screened by plaque hybridization using standard methods. Phagemid clones were recovered from λZAPII clones per the manufacturer's instructions.

Plant growth conditions and inoculation. Inoculation of endophyte-free seedlings of perennial ryegrass (*Lolium perenne* cv. Nui) was carried out using the method of Latch and Christensen (30) with mycelium from 7- to 14-day-old cultures. Plants were tested for endophyte infection by tissue print immunoblotting (24) as described in reference 61.

Molecular biology. Fungal genomic DNA was isolated from freeze-dried or frozen mycelium by using the methods described in references 1 and 11. Plasmid DNA was isolated and purified using a QIAGEN (Hilden, Germany) plasmid mini kit. Lambda DNA was isolated using a standard method (43).

PCR products amplified with *Taq* DNA polymerase (Invitrogen) were routinely cloned into pCR2.1-TOPO (Invitrogen) and transformed into *E. coli* Top 10.

For Southern analysis, genomic digests were transferred to positively charged nylon membranes (Hybond-N+; Amersham) by capillary transfer. Filters were probed with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham)-labeled probes. DNA was labeled by primed synthesis with the Klenow fragment and a RadPrime Kit (Invitrogen). Hybridizations were carried out at 65°C overnight in Church and Gilbert buffer (43) followed by three washes with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, the first at room temperature for 5 min and the second and third at 50°C for a total of 1 h. Blots were subsequently exposed to X-ray film.

Large DNA fragments were separated using contour-clamped homogeneous electric field (CHEF) electrophoresis (16) in an agarose gel (Bio-Rad; pulsed-field grade) using a CHEF-DR III electrophoresis unit at 14°C in 0.5× Tris-borate-EDTA buffer (43) at 6 V/cm using switch times of 50 to 90 s for 22 h. Chromosomal DNA was embedded in agarose and digested as described in reference 62.

Semiquantitative PCR analysis. RNA was isolated from frozen fungal mycelia or pseudostem tissue of infected perennial ryegrass by using TRIzol reagent (Invitrogen). DNA was removed by incubating 1-μg aliquots of total RNA with 10 U of RNase-free DNase I (Roche) for 30 min at 37°C, followed by 10 min at 75°C. RNA was reverse transcribed at 60°C for 60 min in a reaction volume of 20 μl containing 1× reaction buffer, 10 mM dithiothreitol, 1 mM (each) deoxynucleoside triphosphates, 2.5 μM oligo$(dT)_{20}$, 2 U of RNase inhibitor, and 0.75 U of ThermoScript reverse transcriptase (Invitrogen). Gene-specific amplification from cDNA was carried out in a 20-μl volume containing 1× *Taq* polymerase buffer, 50 μM (each) deoxynucleoside triphosphates, 200 nM (each) primers, and 0.5 U of *Taq* polymerase (Invitrogen).

The thermocycling conditions used for semiquantitative PCR were one cycle at 94°C for 2 min; 30 cycles at 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s; and a final incubation at 72°C for 5 min. Primer pairs used were lpsBq-F/lpsBq-R (*lpsB*), DamP81/DamP82 (*easH*), DamP83/DamP84 (*easA*), DamP85/DamP86 (*easG*), DamP87/DamP88 (*easF*), and DamP89/DamP90 (*easE*); for descriptions, see Table S1 in the supplemental material.

Preparation of deletion construct. The targeted deletion construct, pDF6, was constructed using a MultiSite Gateway (Invitrogen) system. Flanking sequences of 3.1 kb, 5′ and 3′ of *lpsB*, were amplified by PCR with attB1- and attB4-tailed primers (KOLEnd-F/KOLEnd-R) and attB3- and attB2-tailed primers (KOREnd-F/KOREnd-R), respectively, from *E. festucae* Fl1 genomic DNA. A 4.1-kb fragment containing P_{gpdA} *hph* was amplified from pAN7-1 (39) with attB1- and attB2-tailed primers (Hyg-F/Hyg-R). These fragments were recombined into pDONR P4-P1R, pDONR P2R-P3, and pDONR 221, respectively, using BP Clonase (Invitrogen). The three fragments were recombined into pDEST R4-R3 in a three-way recombination reaction using LR Clonase Plus (Invitrogen), resulting in pDF6. The linear product of pDF6 used for transformation was amplified using TripleMaster polymerase (Eppendorf) using conditions recommended by the manufacturer.

Transformation of *E. festucae* protoplasts and molecular analysis of transformants. Protoplasts of *E. festucae* Fl1 were prepared using the method of Young et al. (60) except that 10 mg/ml Glucanex (Chemcolour Industry) was used to digest the cell walls. Protoplasts were either transformed with 5 μg of a linear PCR product of pDF6 for targeted gene replacement or cotransformed with 5 μg of pDF1 and 2.5 μg of pII99 (36) for complementation. Transformants were

selected on RG medium (PD plus 0.8 M sucrose) containing either hygromycin (150 μg/ml) for replacement constructs or Geneticin (220 μg/ml) for complementation. To obtain clonal isolates, the resulting transformants were purified by subculturing three times as described by Young et al. (61).

Transformants were screened by PCR for homologous recombination of the replacement construct with primers lpsBKOs-F and lpsBKOs-R, which annealed to sequences in the replacement construct on either side of the *hph* cassette, amplifying a 400-bp product in wild-type *E. festucae* and a 4-kb product in the mutant. The mutant strain chosen for analysis was further screened by Southern blot analysis as described above.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. Pseudostems (two to three per plant, approximately 35 mm in length) were collected from perennial ryegrass plants that were either endophyte-free or infected with *E. festucae* Fl1, an *lpsB* mutant, or complemented strains, after 8 weeks of growth in the greenhouse. Samples were also collected from perennial ryegrass cv. Rosalin infected with *Neotyphodium* sp. strain Lp1 (37) as a reference association. Samples (approximately 50 mg) were extracted by maceration with a ceramic bead in a tissue disrupter (FastPrep FP120; Savant) for 60 s (setting 4) in 500 μl methanol–1% aqueous acetic acid (1:1) containing 0.112 μg/ml ergotamine tartrate as the internal standard. Subsamples (15 μl) of the supernatant were eluted through a C_{18} Luna column (Phenomenex) (150 by 2 mm; column particle size, 5 μm) at a flow rate of 200 μl/min using a Surveyor HPLC (Thermo Finnigan) with a solvent gradient, starting with 5% MeCN:95% H_2O (containing 5 mM ammonium acetate) for 5 min and then increasing to 50% MeCN over 38 min followed by a column wash at 100% MeCN. To limit carry-over between samples, four wash cycles were carried out between each injection. Cross-contamination between samples was very low but detectable (ca. 1/1,000), and endophyte-free samples were interspersed between endophyte-infected samples to avoid ambiguity. Mass spectrometry was carried out by electrospray ionization in the positive mode with a linear ion trap mass spectrometer (LTQ; Thermo Finnigan). The spray voltage was 5.0 kV, and the capillary temperature was 275°C. The flow rates of sheath gas and auxiliary gas were set to 20 and 10 (arbitrary units), respectively. Metabolites were identified by comparisons of elution times and product ion spectra from collision-induced dissociations of selected precursor ions (35% collision energy) with authentic standards or, in the case of lysergyl-alanine and its isomer and 6,7-secolysergine, with previously characterized metabolites of *Neotyphodium* sp. strain Lp1 (37). Measurements were carried out by selective reaction monitoring of the transitions from *m/z* 269 to 223 (lysergic acid and isolysergic acid), *m/z* 340 to 223 (lysergyl-alanine and isolysergyl-alanine), *m/z* 268 to 223 (ergine and erginine), *m/z* 241 to 210 (6,7-secolysergine and putative isomers), *m/z* 534 to 268 (ergovaline and ergovalinine), and *m/z* 582 to 268 (ergotamine and ergotaminine).

DNA sequencing and bioinformatics analyses. DNA fragments were sequenced by the dideoxynucleotide chain termination method using Big-Dye 3.1 chemistry with oligonucleotide primers (Invitrogen), either M13 forward and reverse or specific to *N. lolii* or *E. festucae* genomic sequences. Products were separated with an ABI Prism 3730 sequencer (PerkinElmer).

Sequence data were assembled into contigs and analyzed using Vector NTI suite 9 (Invitrogen). Sequence comparisons with public databases were performed via the Internet at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/), by employing BLASTN, BLASTX, and BLASTP algorithms.

Repeat sequences were identified and characterized using MEME (Multiple EM for Motif Elicitation) (3) and the etandem and einverted algorithms of EMBOSS (41).

Nucleotide sequence accession number. The *EAS* cluster sequence isolated in this study has been submitted to the DDBJ/EMBL/GenBank databases (accession number EF125025).

RESULTS

Isolation and analysis of a putative gene cluster for ergovaline biosynthesis. PCR amplification of *N. lolii* NRPS adenylation domains in a previous study identified a clone, ps12, that was preferentially expressed in planta (48). To clone the full-length gene, the ps12 PCR product was used to probe genomic libraries. This region of the genome was found to be underrepresented in all libraries screened. Just two clones (pDF1 and pDF2), from ~80,000 recombinant phages plated, were isolated from an *N. lolii* Lp19 small-insert λZAPII library, and no clones were identified for *N. lolii* Lp19 bacterial artificial chromosome or *E. festucae* Fl1 cosmid and fosmid libraries. The sequence of pDF1 and pDF2 covered 9.8 kb (Fig. 1), and bioinformatics analysis revealed two open reading frames (ORFs) encoding predicted proteins with 49% and 48% identity, respectively, to genes for a single-module NRPS, *lpsB* (formerly *cpps2*), and an oxidoreductase, *easE* (formerly *cpox1*), found in a gene cluster for ergopeptine biosynthesis in *C. purpurea* (18). These results suggested that the two putative genes, designated *lpsB* and *easE* following the convention of Schardl et al. (46), encode genes for the biosynthesis of ergovaline, the predominant ergopeptine product in *N. lolii*.

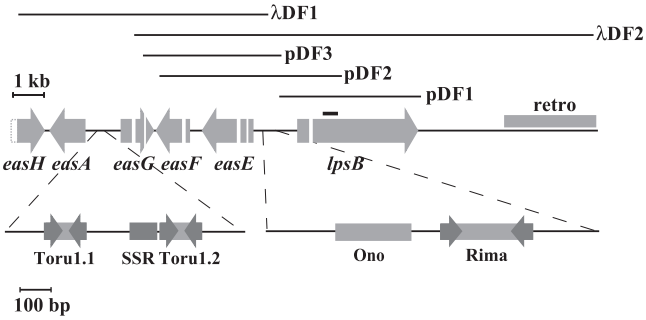

FIG. 1. Physical map and clones covering the *N. lolii EAS* locus. Putative genes are shown as arrows indicating the direction of transcription. Introns are shown as gaps. The solid line above *lpsB* denotes the ps12 sequence used to probe genomic libraries. "Retro" denotes two highly degenerate nested long terminal repeat retrotransposons. Regions containing putative nonautonomous transposable elements are expanded. SSR, simple sequence repeat (eight times 10 bp).

An analysis of the predicted amino acid sequence of LpsB revealed the single module to contain an adenylation domain, a thiolation domain, and a condensation domain. As the LpsB NRPS is predicted to interact with a second NRPS, LpsA, in order to catalyze the formation of lysergyl peptide lactam, we aligned the *N. lolii* and *C. purpurea* LpsB carboxy-terminal sequences with those of bacterial NRPSs involved in multienzyme complexes (data not shown). No alignment was found with COM domains required for protein-protein interaction in bacterial systems.

To extend the cluster, additional clones were isolated by screening λZAPII and λGEM-12 libraries. No clones could be isolated from the λZAPII library using a probe to the right end of the cluster amplified with primer set DamP10/DamP11, and just one clone, pDF3, was isolated using a probe to the left end of the cluster amplified with primers DamP21/DamP22 that extended the sequence only 791 bp. Left-end sequence was present, albeit underrepresented, in the λGEM-12 library with four clones, λDF1-4, isolated from the 110,000 recombinant phages plated. The four clones spanned 19.4 kb and extended the previously isolated sequence by 4.1 kb at the left end and 5.5 kb at the right end (Fig. 1).

Bioinformatics analysis of the 4.1-kb left-end sequence identified four putative genes encoding an oxidoreductase (*easA*), two conserved ORFs with unknown functions (*easF* and *easG*), and a dioxygenase gene (*easH*) (Table 1 and Fig. 1). The *easH* gene was truncated at the 5′ end in a chimeric lambda clone, λDF1. Each of the additional putative genes in the *EAS* cluster had homologues in the *C. purpurea* and *A. fumigatus* ergot

TABLE 1. Bioinformatics analysis of genes within the *N. lolii EAS* cluster[a]

Gene	Introns (bp)	Size (aa)	BLASTP E value		Function or putative function
			C. purpurea	*A. fumigatus*	
lpsB	75	1,352	0.0	—	Single-module NRPS
easE	75, 60	605	e-123	e-113	Reductase/dehydrogenase
easF	69	344	e-118	e-115	Methyltransferase
easG	78, 53	309	4e-78	1e-64	Reductase/dehydrogenase
easA	—	380	e-152	57e-124	Reductase/dehydrogenase
easH	—	188	ND	0.53	Oxygenase/hydroxylase

[a] *easH* is truncated at the 5′ end in λDF1; therefore, only a partial sequence was studied. ND, not determined as the *C. purpurea easH* gene sequence is not available in public databases; —, absent; aa, amino acids.

alkaloid gene clusters (Table 1). Sequence analyses of each of the three gene clusters (Fig. 2) showed substantially different gene orders among the three clusters.

The 5.5-kb right-end sequence was extremely AT rich (76%) compared with the *eas* genes (50%). A BLASTX analysis of this region indicated that this sequence is derived from two nested retrotransposons but is highly degenerate, with no evidence of intact ORFs. This degeneracy is probably a result of repeat-induced point mutation (RIP), which results in C:G-to-A:T transitions (12) and was previously observed in two epichloë retroelements, Tahi and Rua, associated with the *LTM* gene cluster (61).

Linkage of the *EAS* gene cluster with *dmaW* and *lpsA* and identification of *cloA*. Two genes for ergovaline biosynthesis have been previously characterized: *dmaW*, a DMAT synthase-encoding gene required for the first committed step in ergot alkaloid biosynthesis (52), and *lpsA*, a gene encoding a trimodular NRPS that, along with a second NRPS, predicted to be LpsB, is required for formation of lysergyl peptide lactam (38). To test the hypothesis that these genes were physically linked with the gene cluster isolated in this study, we screened the λZAPII library for clones containing these genes. No clone could be isolated for *dmaW*; however, a BLASTX analysis of the *Neotyphodium coenophialum dmaW-2* flanking sequence in GenBank (accession number AY259839.1) revealed the presence of a P450 monooxygenase gene orthologous with the *C. purpurea cloA*-encoded monooxygenase required for conversion of elymoclavine to D-lysergic acid (26) (Fig. 3). PCR analysis showed *cloA* to be present in *N. lolii* Lp19 and *E. festucae* Fl1 but directly upstream of *dmaW* only in *E. festucae* (data not shown).

One clone that was isolated for *lpsA* contained 1,204 bp of unique sequence 5′ of the *lpsA* sequence deposited in GenBank (accession AF368420). This sequence contained 655 bp of a Tahi long terminal repeat retrotransposon relic at the 5′ end. The AT richness (76%) of this element precluded further chromosome walking. We therefore performed Southern analyses of NotI digests of *N. lolii* Lp19 and *E. festucae* Fl1 genomic DNA (Fig. 4). Probes for *lpsB*, *lpsA*, and *dmaW* each hybridized to a 114-kb fragment in *E. festucae* and a 340-kb fragment in *N. lolii*, indicating that *dmaW* and *lpsA* were linked with the *EAS* cluster in each species.

Transposable elements associated with the *EAS* cluster. BLASTN and MEME analyses of the *EAS* cluster revealed the presence of putative nonautonomous transposons between *easG* and *easA* and between *easE* and *lpsB* (Fig. 1). Two elements in the intergenic region between *easG* and *easA* have the characteristics of a miniature inverted repeat transposable element (MITE) (21). They are 82% identical, are 139 and 141 bp in size, respectively, and have 61-bp imperfect terminal inverted repeats (TIRs) and a putative AT target site duplication (TSD). The 24 bp between the TIRs is AT rich and has no similarity with any sequence in the public databases. We named the element Toru (Maori for three) following the convention adopted for naming the two retrotransposons in the *LTM* cluster called Tahi and Rua (one and two) (61).

A BLASTN analysis showed Toru elements to be present upstream of epichloë *dmaW* (Fig. 3) and also in other epichloë biosynthetic gene clusters (see Table S2 in the supplemental material). Analysis of the "extra" sequence between *N. coenophialum dmaW-1* and the Toru MITEs, compared with *dmaW-2* and *Neotyphodium* sp. strain Lp1, revealed a putative

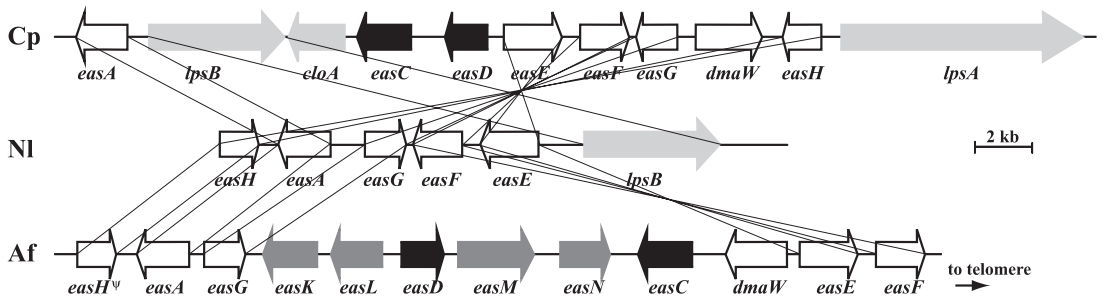

FIG. 2. Comparative *eas* gene order among *N. lolii*, *C. purpurea*, and *A. fumigatus*. Cp, *C. purpurea*; Nl, *N. lolii*; Af, *A. fumigatus.* Open arrows, genes shared between each organism; black arrows, genes proposed to be required for ergot alkaloid synthesis but not yet identified for *N. lolii*; light gray arrows, genes found in *N. lolii* and *C. purpurea* but not *A. fumigatus*; dark gray arrows, genes found only in *A. fumigatus*. *N. lolii dmaW*, *cloA*, and *lpsA* are not shown, as their locations relative to the *EAS* cluster are not known.

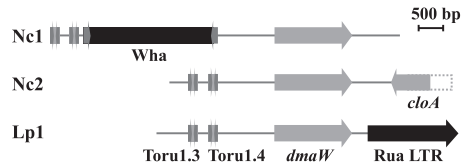

FIG. 3. Sequence diversity at *dmaW* loci. Nc1, *N. coenophialum dmaW* locus 1 (accession number AY259838); Nc2, *N. coenophialum dmaW* locus 2 (accession number AY259839); Lp1, *Neotyphodium* sp. strain Lp1 *dmaW* locus (accession number AY259837). LTR, long terminal repeat.

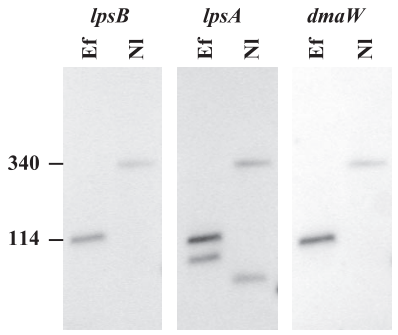

FIG. 4. Southern blot analysis indicating linkage of *lpsB*, *lpsA*, and *dmaW*. *N. lolii* Lp19 (Nl) and *E. festucae* Fl1 (Ef) genomic DNA was digested with NotI, separated by CHEF pulsed-field gel electrophoresis, and transferred to a nylon membrane. The blot was hybridized with ^{32}P-labeled probes to *lpsB* (pDF2), *lpsA* (amplified with primers lpsA-F and lpsA-R), and *dmaW* (amplified with primers dmaWq-F and dmaWq-R). The *lpsA* probe contains a NotI site and hence hybridizes to two bands.

type 2 Mutator-like element relic, Wha (Maori for four) (Fig. 3). Wha has 95-bp TIRs and a 10-bp putative TSD. The 2,413 bp between the putative TIRs was highly degenerate (75% AT), probably due to RIP, and shared 32% identity with a 312-amino-acid putative protein (CHGG_06856) from *Chaetomium globosum*, with several closely related proteins in the *C. globosum* genome. When submitted for BLASTX analysis itself, this sequence had 31% identity with a transposase from the Hop Mutator-like element in *Fusarium oxysporum* (13).

A second MITE-like element, Rima (Maori for five), is present between the *lpsB* and *easE* genes. This element is 295 bp in size and has two 63-bp imperfect TIRs, a putative TA TSD, and AT-rich noncoding sequence between the TIRs. No other copies of Rima were found in GenBank by BLASTN analysis. Southern analysis showed the element to be single copy in the *E. festucae* Fl1 and *N. lolii* Lp19 genomes (data not shown); however, this may be due to degeneracy of other Rima elements, as is observed with Toru elements.

A third putative nonautonomous repeat element is present 97 bp from Rima. This element, termed Ono (Maori for six), is 236 bp long and is 82% and 66% identical to sequences upstream of *ltmM* and *ltmE*, respectively, in the *LTM* gene cluster. Of the three Ono elements, only the *ltmM*-associated element has a putative TSD of 7 bp. This element had been identified previously as a 115-bp sequence shared between the *ltmM* and *ltmE* promoter regions and was described as possibly being a relic of a short interspersed nuclear element (62).

Functional analysis of *lpsB*. To confirm that *lpsB* was required for ergovaline biosynthesis, deletion of a 276-bp region of the adenylation domain was performed by the introduction of a targeting construct containing 6 kb of homologous sequence and a hygromycin resistance cassette (Fig. 5A). A PCR-generated linear construct was transformed into *E. festucae* Fl1 protoplasts. *E. festucae*, the sexual progenitor of *N. lolii* (35), was used rather than *N. lolii* because of its genetic tractability (44, 61). Transformants that grew in the presence of hygromycin were screened by PCR, and 15% of these (7 of 48 screened) showed a replacement event (data not shown). An *lpsB* mutant, DFM3, was confirmed to be a replacement by Southern analysis (Fig. 5B). This mutant showed no difference in morphology or growth rate in culture compared with the parent isolate and showed a wild-type plant-interaction phenotype, demonstrating that disruption of *lpsB* has no effect on the symbiosis under normal growth conditions.

LC-MS/MS with selected reaction monitoring was performed to determine the effect of the *lpsB* mutation on ergot alkaloid production (Fig. 6) (see Table S3 in the supplemental material). The structures of lysergic acid, lysergyl alanine, and ergovaline are shown in Fig. 6. Ergovaline and its stereoisomer ergovalinine were not detected in samples from symbiota containing the *lpsB* mutant compared with the wild type, confirming the expected requirement of *lpsB* for ergovaline biosynthesis. Lysergic acid and its C-8 stereoisomer were observed to accumulate to elevated levels in *lpsB* mutant symbiota compared to wild-type associations. Lysergyl alanine, an amide of lysergic acid previously identified by Panaccione et al. (37) as a missing compound in an *lpsA* mutant of *Neotyphodium* sp. strain Lp1, was also identified together with its C-8 stereoisomer in symbiota containing *E. festucae* strain Fl1 but was absent in *lpsB* mutant symbiota. Synthesis of ergine, another lysergic acid amide, was also shown to be blocked by *lpsA* mutation in *Neotyphodium* sp. strain Lp1 (37). While no samples of *E. festucae lpsB* mutant-infected symbiota contained ergine, production of ergine was not always detected in wild-type symbiota. The novel clavine compound 6,7-secolysergine, observed to accumulate in the *Neotyphodium* sp. strain Lp1 *lpsA* mutant (37), was not observed at significant levels in any *E. festucae* association. However, an unknown peak detected by LC-MS/MS monitoring of the selective reaction characteristic of 6,7-secolysergine (*m*/*z* 241 to 210) did appear to increase in concentration in the *E. festucae lpsB* mutant symbiota (see Table S3 in the supplemental material). Analyses of other clavine intermediate compounds did not show any reduction or accumulation resulting from *lpsB* mutation (data not shown).

In order to complement the *lpsB* mutation, pDF1, containing the wild-type *lpsB* gene and 379 bp of upstream sequence (Fig. 1), was transformed into *lpsB* mutant protoplasts. An arbitrary selection of these transformants (DFM7, DFM8, and DFM9) was used to infect perennial ryegrass seedlings, and the ergot alkaloid phenotype of these associations was determined by LC-MS/MS (Fig. 6; see Table S3 in the supplemental material). These strains were able to synthesize ergovaline and lysergyl alanine and, on occasion, ergine.

Expression analysis. Ergovaline is synthesized preferentially during biotrophic growth. To test whether each of the *eas* genes is preferentially expressed in planta, semiquantitative reverse transcription (RT)-PCR analysis was performed with

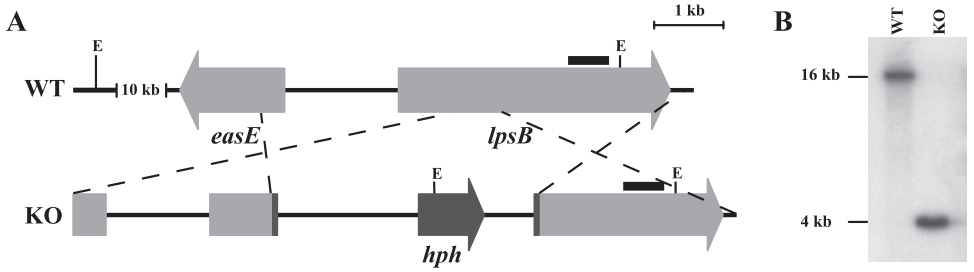

FIG. 5. Targeted gene replacement of *lpsB*. (A) Diagrammatic representation of homologous recombination at *lpsB*. The wild-type (WT) gene locus and targeted replacement construct (KO) are shown. The solid line above *lpsB* represents the PCR probe amplified with primers KOprobe-F and KOprobe-R, used for the results shown in panel B. E, EcoRI site. (B) Southern blot analysis. Wild-type and *lpsB* mutant DFM3 (KO) genomic DNA was digested with EcoRI and transferred to a nylon membrane, which was hybridized with the probe shown in panel A.

RNA extracted from *L. perenne* Nui infected with *E. festucae* Fl1 and from *E. festucae* Fl1 grown axenically in PD broth (Fig. 7). To normalize the fungal RNA levels in each sample (48), cDNA derived from mycelia grown in PD broth was serially diluted to 1/100. At this dilution, a band with an intensity similar to that of the in planta cDNA was amplified by PCR using primers for *E. festucae* Fl1 *tubB*. Each gene in the *EAS* cluster was strongly expressed in planta but either weakly expressed, in the cases of *easG* and *easH*, or not at all expressed in axenic culture when grown in complex media.

To test the hypothesis that ergovaline genes may be subject to catabolite repression in culture, we isolated RNA from *E. festucae* Fl1 grown in carbon-, nitrogen-, and phosphate-limited minimal media, as well as from PD media and infected perennial ryegrass. This RNA was tested for *lpsB* expression by real-time RT-PCR, and no expression could be detected under any of the growth conditions. Transfer of *E. festucae* Fl1 grown in PD media to a perennial ryegrass extract for 30 min also failed to induce *lpsB* expression.

DISCUSSION

We describe here the isolation of a cluster of genes proposed to be required for ergot alkaloid biosynthesis in the grass endophyte *N. lolii*. Molecular cloning and functional analysis of a plant-regulated NRPS indicates that it is likely to be an orthologue of the *C. purpurea lpsB* gene, encoding the LpsB NRPS required for synthesis of ergovaline. Isolation and analysis of sequences flanking *lpsB* identified a cluster of genes homologous to ergot alkaloid biosynthetic gene clusters found

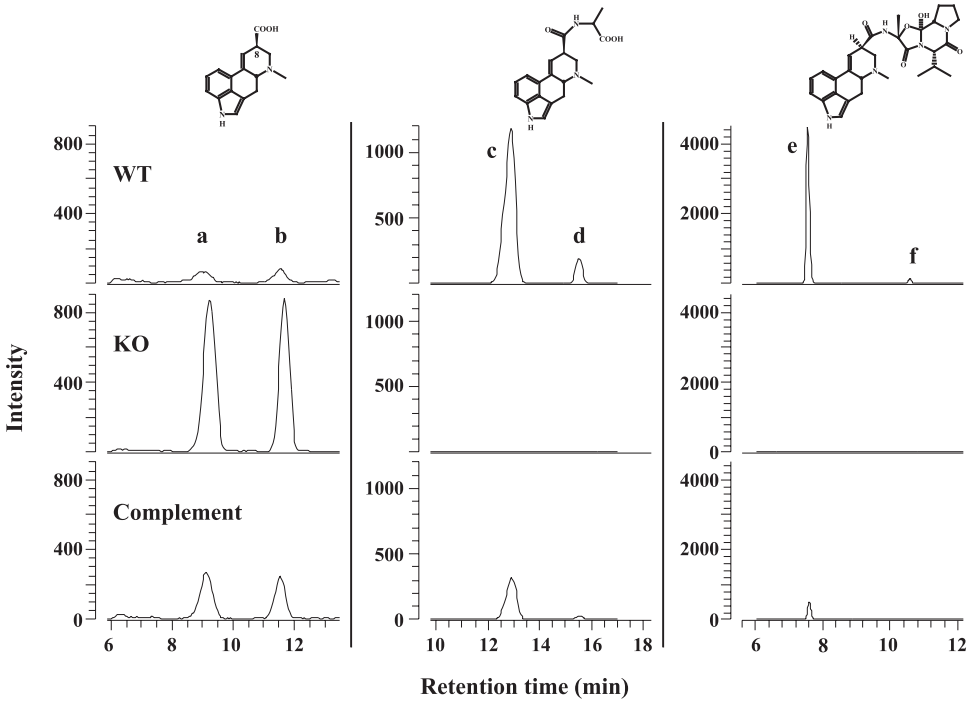

FIG. 6. Extracted ion chromatograms from LC-MS/MS of extracts of perennial ryegrass plants infected with wild-type *E. festucae* Fl1 (WT), *lpsB* mutant DFM3 (KO), or DFM3 transformed with pDF1 (Complement), showing accumulation of lysergic acid (shown above; peak a) and isolysergic acid (peak b) (*m/z* 268 to 223), lysergyl-alanine (peak c) and isolysergyl-alanine (peak d) (*m/z* 340 to 223), and ergovaline (peak e) and ergovalinine (peak f) (*m/z* 340 to 223). Relative abundance values are on the same (arbitrary) scale for each extract.

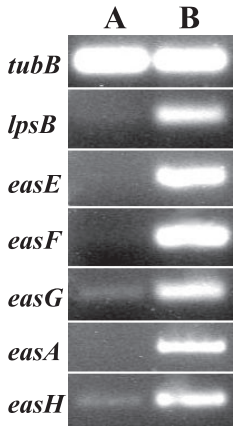

FIG. 7. Expression analysis of genes in the *EAS* cluster. RT-PCR was performed with RNA extracted from *E. festucae* Fl1 grown axenically in PD medium (A) and *L. perenne* infected with *E. festucae* Fl1 (B). cDNA prepared from cultured mycelium was diluted 1/100 to compensate for the biomass differences in planta.

in *C. purpurea* (18, 25, 53) and *A. fumigatus* (19, 54). With the exception of *easH*, which is likely to be a pseudogene in *A. fumigatus* (46), the genes common to all three clusters are proposed to encode enzymes for the early steps in the ergot alkaloid biosynthetic pathway. Genes that are shared by *C. purpurea* and *N. lolii* but absent from *A. fumigatus*—*lpsA*, *lpsB*, and *cloA*—have been shown to be required for steps leading to ergopeptines (18, 26, 38). Just two genes, *easC*, a predicted catalase-like gene, and *easD*, a predicted oxidoreductase, common to *C. purpurea* and *A. fumigatus* clusters remain to be identified for *N. lolii*. Sequencing of the *E. festucae* strain E2368 genome is currently under way (C. Schardl, personal communication), and the results will facilitate future cloning of these remaining genes.

While gene sequence is relatively highly conserved between each of the three ergot alkaloid gene clusters, there are several differences in gene order, and the *N. lolii* cluster is more complex in structure and organization. The transposon insertions in the *EAS* cluster have generated several repeat sequences that could be signatures of previous recombination events that led to these rearrangements. The presence of putative transposons in the intergenic regions of each of the fully sequenced genes in the *N. lolii* cluster that are rearranged compared with *C. purpurea* supports this hypothesis. The *EAS* cluster is linked with *dmaW* and *lpsA* in *N. lolii* and *E. festucae* on substantially differently sized NotI fragments, indicative of the presence of additional transposon blocks in *N. lolii*, as is the case at the *LTM* locus, which is associated with three large blocks of repeat sequences in *N. lolii* and just two in *E. festucae* (62). The abundance of transposon relic sequences adds to the evolutionary potential of the *EAS* cluster. In *Magnaporthe oryzae*, transposable element clusters correlate with increased recombination rate, loss of synteny, gene duplication, and sequence divergence from orthologous genes (51). Inter- and intrachromasomal recombination mediated by these repeat sequences in the *N. lolii* genome could be important in generating genetic diversity.

The predicted *N. lolii* LpsB enzyme has a modular structure similar to that of the orthologous *C. purpurea* enzyme, including an adenylation domain, thiolation (peptidyl carrier protein) domain, and condensation domain. LpsB is unusual among fungal NRPSs in that it forms a multienzyme complex with LpsA (42). Multienzyme NRPSs are common in prokaryotes, but the LpsA/LpsB system is the only described example in the fungal kingdom, where NRPSs are otherwise found on a single large multimodular polypeptide (55). There is a condensation domain found at the carboxy end of the LpsB protein and also a partial condensation domain at the amino-proximal end of the predicted *N. lolii*, but not *C. purpurea*, LpsA enzyme (20). Whether the partial LpsA condensation domain plays a role in the condensation of the lysergyl-alanine peptide bond in the *N. lolii* enzyme system remains to be determined. A feature of multienzyme NRPSs in prokaryotic systems is the presence of recently described COM domains, which are required for specific protein-protein recognition (27). Alignment of the carboxy end of the predicted *N. lolii* and *C. purpurea* LpsB sequences with bacterial donor COM domains showed that the TPSD motif present at the junction between epimerization and COM domains is absent, a result not totally unexpected given that the C terminus of LpsB is preceded by a condensation domain. The amino-terminal LpsA sequence from *N. lolii* and the LpsA-1 and LpsA-2 sequences from *C. purpurea* are also dissimilar to the bacterial acceptor COM domain consensus sequence and, interestingly, to each other. With no other fungal multienzyme NRPS systems yet described, functional analysis is required to identify the residues important for LpsB-LpsA protein-protein interaction.

To confirm the role of *lpsB* in the formation of ergovaline, targeted disruption was performed with *E. festucae*, the more genetically tractable sexual progenitor of *N. lolii*. Plants infected with this mutant were unable to synthesize ergovaline, while complementation with a fragment containing the *lpsB* gene sequence restored this ability, confirming that *lpsB* is required for ergot alkaloid biosynthesis. While complementation did not restore ergovaline production to wild-type levels, possibly due to the length of upstream sequence (379 bp) or position effects of the ectopically transformed constructs, complemented strains were clearly able to synthesize ergovaline. A previous analysis of an *lpsA* mutant of *Neotyphodium* sp. strain Lp1 had shown a loss of lysergic acid amides lysergyl alanine and ergine along with ergovaline, suggesting that they are derived from either the lysergyl peptide lactam or ergopeptine (37). Our results, showing that synthesis of lysergyl alanine is also blocked by mutation of *lpsB*, support this idea. Although production of ergine by wild-type *E. festucae* symbiota was not always detected, its absence from all plants infected with the *lpsB* mutant strain and occurrence in some plants with the complemented strains suggest that *lpsB* is required for its synthesis. As with the *lpsA* mutant of *Neotyphodium* sp. strain Lp1, accumulation of the major identified clavine alkaloids did not appear to be affected by mutation of *lpsB.* An increased concentration of an unidentified minor clavine was noted but, interestingly, this appears to be an isomer of the clavine tentatively assigned the structure 6,7-secolysergine, which increased in concentration in the *Neotyphodium* sp. strain Lp1 *lpsA* mutant compared with the wild type (37).

Ergot alkaloids are specifically produced during biotrophic growth of epichloë endophytes. Attendant with this, the *eas*

genes are all expressed in planta but not under any axenic culture condition tested. Ergot alkaloid biosynthetic genes in *C. purpurea* cultures are repressed by high phosphate levels (18, 25). However, our results suggest that in *E. festucae*, at least the *lpsB* gene is not derepressed in culture by phosphate, carbon, or nitrogen catabolite starvation. These results suggest that specific plant conditions may be required for induction. Several genes that are specifically expressed in response to plant signals have been identified for plant-associated fungi (4, 5, 49, 58); however, it was only recently, when Yang et al. (59) showed that the *pelD* gene of *Nectria haematococca* was expressed in response to homoserine and asparagine, that a specific plant factor responsible for expression of one of these genes was identified. The inability of *E. festucae* Fl1 to induce *lpsB* expression in the presence of a plant extract suggests that the inducing factor was not stable or, alternatively, that specific signaling between the epichloë endophyte and the grass host occurs.

Chromatin remodeling as a method of coordinate gene regulation is a possible factor causing selection pressure for secondary metabolite genes to be clustered. The presence of transposons at the *EAS* locus, particularly AT-rich, RIP-degenerated sequence, a potent inducer of DNA methylation in *Neurospora crassa* (47), suggests that regulation at the level of chromatin may be important for *eas* genes. The identification in *Aspergillus nidulans* of a putative histone methyltransferase, LaeA, that is a global secondary metabolite gene regulator (6), and the finding that transfer of a housekeeping gene to a location within the sterigmatocystin gene cluster partially silences expression (7), supports a gene silencing mechanism. Experiments to determine *cis* and *trans* factors required for transcription of *eas* genes and the effect of genomic location are currently under way. Identifying the regulatory mechanisms that activate the expression of the *eas* genes in planta and determining whether there is some commonality with mechanisms for activating other plant-induced endophyte genes will be of considerable future interest.

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